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Missing the target: DNAk is a dominant epitope in the humoral immune response of channel catfish (*Ictalurus punctatus*) to *Flavobacterium columnare*

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ABSTRACT

Vaccination remains a viable alternative for bacterial disease protection in fish; however additional work is required to understand the mechanisms of adaptive immunity in the channel catfish. To assess the humoral immune response to *Flavobacterium columnare*; a group of channel catfish were first immunized with *F. columnare* LV-359-01 cultured in iron-depleted media, before being challenged with wild type *F. columnare* LV-359-01. The immunization protocol did not confer increased protection against *F. columnare*; however both control and immunized responders generated serum and skin IgM antibodies against *F. columnare* proteins. Western blot analyses of individuals from both groups showed that IgM antibodies were generated to the same 70 kDa extracellular protein, which was identified to be the bacterial chaperonin protein DNAk. Antibodies generated were cross reactive to DNAk proteins found in other gram negative bacteria. Our data suggests that DNAk is the dominant epitope in the channel catfish B-cell response to *F. columnare*.

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1. Introduction

Flavobacterium columnare, the etiological agent of columnaris disease, is a ubiquitous and opportunistic fish pathogen that is highly transmissible and causes widespread mortality throughout the aquaculture industry [20,48]. Strategies to combat columnaris infections, going back nearly a century, have included lowering rearing density, salt baths, acid baths, and chemical therapeutants [54]. However, these approaches have failed to reduce columnaris disease incidence, as they are largely reactive measures implemented after the onset of disease [54]. More recently, the effective management of columnaris has been further constrained by ever evolving regulatory burdens associated with new and existing treatment compounds, and emerging concerns over antibiotic resistance [10]. Immunization-based preventative strategies remain a viable and promising alternative for bacterial disease protection in fish; and there has been no shortage of work done to develop and to evaluate immunogens for use in vaccination against columnaris disease [29,30,34–36].

Despite tremendous research effort in this area, findings regarding efficacy have been mixed, and are likely due to a number of factors including the parental strain of the isolate used [29], species and age of the immunized fish, the preparation/engineering of the vaccine candidate, and disparities in vaccination doses and durations. Nevertheless, it is becoming increasingly apparent that the use of live modified or attenuated columnaris vaccines may offer potential for use in aquaculture settings. Generally, and in contrast to killed bacterins, advantages of live attenuated vaccines include the stronger induction of both humoral and cell mediated immunity, require smaller doses to induce a robust and long-lasting immune response, entail minimal to no adjuvants, and feature a more natural means of exposure (i.e., immersion or oral routes); which would be more amenable to the large-scale immunizations required in settings of aquaculture [43].

While our level of understanding is growing, expansive knowledge gaps remain; especially lacking is a comprehensive view of species-specific immune responses to immunogens, and how these responses govern host protection. One such species in

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particular need of further study is the channel catfish, the predominant warmwater aquaculture species in the United States, and a highly susceptible host to columnaris disease. Due to its commercial importance, the channel catfish is one of the better-studied immune models among teleost fish with fundamental discoveries on their immunological form and function dating back more than three decades [1,8,15,22,25–28]. Recently, the rate of discovery has been accelerated in the catfish model system with the rise of next generation sequencing platforms. Transcriptomic studies of target mucosal tissues in the context of experimental disease challenges have helped dissect the initial stages of columnaris pathogenesis [4,31,39,42,47]. Even so, insight into the cellular and humoral effectors, at the level of protein, that modulate vaccine success is needed to make meaningful improvements in columnaris prevention.

Previously, we demonstrated that the *F. columnare* isolate LV-359-01 grew poorly under iron-limited conditions and was significantly less virulent as compared to when cultured under normal conditions [5]. Building upon these previous findings, and borrowing from prior work in *Flavobacterium psychrophilum* where an isolate negatively impacted by iron deprivation showed potential as a vaccine candidate [2], here, we set out to evaluate the utility of iron restriction as a means by which to develop a putative vaccine for columnaris disease. Unexpectedly, we failed to confer protection using an immersion-based immunization protocol with the iron-restricted isolate. However, further investigation revealed a robust and highly specific antibody response, displayed by both serum and skin, to a single *F. columnare* protein identified to be a ~70 kD heat shock protein, orthologous to *Escherichia coli* DNAk. In the following report, we describe the significance of this immunodominant protein and discuss its putative role in hindering appropriate and protective immune responses in the channel catfish host.

2. Materials and methods

2.1. Bacterial culture and fraction preparations

Different bacterial isolates were utilized throughout the study; *F. columnare* LV-359-01 and LSU-066-04, *Escherichia coli* ATCC 25922, *Aeromonas hydrophila* 0702 and *Edwardsiella ictaluri* S97-773. All isolates were retrieved from frozen glycerol stocks that were stored at −80 °C and streaked onto *F. columnare* Growth Medium (FCGM) [9,14]; or tryptic soy agar with 5% sheep's blood (ThermoFisher, Waltham, MA). After 48 h of growth at 28 °C, isolates were dislodged from the agar using a sterile loop and inoculated into 50 mL of FCGM or brain-heart infused medium (Becton Dickinson, Sparks, MD) and incubated in broth at 28 °C for 24 h. The bacterial suspensions were then spun using an Eppendorf 5810R centrifuge at 6320*g* for 20 m. The extracellular portion (ECP) was poured off into a new tube and spun again for an additional 10 m. The bacterial pellets were resuspended in 5 mL of 1× PBS and sonicated on a setting 7 for 5 m in a Powersonic Model (Crest Ultrasonics, Trenton, NJ). The ECP was poured off and concentrated using 3KMWCO Amicon Ultra-15 centrifugal filter units (EMD Millipore, Billerica, MA). All bacterial pellet and ECP fractions had a 5% (v/v) protease inhibitor cocktail (Sigma Aldrich, St. Louis, MO) added prior to the total protein concentration was estimated using the Coomassie Plus assay kit (Pierce/ThermoFisher, Waltham, MA) with bovine serum albumin (Sigma Aldrich, St. Louis, MO) as the standard. Absorbance was read at a wavelength of 595 nm with a BioTek Synergy H1 plate reader operating under Gen5 software (Winooski, VT). The pellets and ECPs were dispensed as 0.1–1 mL aliquots and kept at −20 °C and thawed as needed and then at 4 °C for no more than 2 weeks. Genbank accession numbers for chaperone protein DNAk: *Escherichia coli* (P0A6Y8), *Flavobacterium columnare* (WP_014165528), *Aeromonas hydrophila* (KLV44233), *Edwardsiella ictaluri* (C5B7L7).

2.2. Immunization and bacterial challenge

Fingerling channel catfish were reared at the Harry K. Dupree Stuttgart National Aquaculture Research Center in Stuttgart, Arkansas, USA. Two hundred fish each, average weight 5 g, were stocked into two 300 L tanks that received filtered well water and aeration from submerged air stones. Fish were offered pelleted catfish feed (35% protein, 2.5% fat; Delta Western, Indianola, Mississippi). There were four groups of channel catfish, non-challenged and non-immunized (N); immunized and non-challenged (I); non-immunized and challenged (C); immunized and challenged (IC) (see Results). To immunize fish through bath immersion, the water level was lowered to 100 L and the fish were exposed to 1 L of *F. columnare* isolate LV-359-01 cultured in iron-depleted media under static conditions for 30 m with a calculated dose of 1.4×10^8 CFU/mL using a drop plate method [5,18]. After fourteen days, control and immunized groups were challenged through bath immersion with wild type LV-359-01 *F. columnare* with a calculated dose of 2.81×10^8 CFU/mL using a drop plate method [18]. For the challenge three replicates of 50 fish (250 g of biomass/tank) were stocked into 18 L tank containing 10 L of filtered well water. Water was provided through the ultra-low-flow water delivery system at a rate of 30 mL/min [3,5]. Fish were not fed on the day of immunization, or on the first day after bacterial challenge. An additional tank containing 50 fish was not challenged and was used as a negative control. Fish were observed twice daily at which time any moribund fish were promptly removed.

Animal care and experimental protocols were approved by the Harry K. Dupree Stuttgart National Aquaculture Research Center Institutional Animal Care and Use Committee and conformed to Agricultural Research Service Policies and Procedures 130.4 and 635.1.

2.3. Blood and skin explant sampling

Twenty fish from the two challenged groups or the non-challenged controls were maintained (post challenge) for forty days in 18 L recirculating aquaria. Fish were then anaesthetized and blood was first collected using a 21-gauge needle from the caudal vein and allowed to clot overnight at 4 °C. Blood samples were centrifuged at 10000*g* for 10 m using an Eppendorf Minispin; the serum (25–100 μL) was removed and stored at −20 °C until needed. After blood collection we proceeded with the preparation of excised skin for tissue culture as described [50,51]. Briefly we wiped down the surface of the skin on both sides three times with a 70% ethanol solution. Then using sterile instruments we dissected two 1.5 mm^2 skin pieces (along the lateral line), washed them with empty Leibovitz's L-15 medium (ThermoFisher, Waltham, MA), and placed them into 400 μL of complete Leibovitz's L-15 medium (10% FBS, penicillin/streptomycin, amphotericin, gentamicin) in a 48-well plate at 28 °C for 24 h. The next day the skin explant tissue culture medium was removed and stored at −20 °C until needed.

2.4. ELISA

We used an indirect ELISA to measure the serum and skin-based IgM antibodies as described with some modifications [37]. Immulon 2HB 96-well plates (ThermoFisher, Waltham, MA) were coated with 100 μl of 10 μg/mL of sonicated *F. columnare* cell pellet in a sodium bicarbonate buffer. Plates were then rinsed three times with 1× PBS with 0.05% Tween-20 (PBST) and then incubated for

1 h in blocking solution (PBST with 5% milk). One hundred μL of serum (1:100) or skin explant (1:2) were further serially diluted out to 1:1600 or 1:32 in 1× PBS on the horizontal axis of an antigen-coated ELISA plate and incubated at room temperature for 1 h. Plates were rinsed as above and 100 μl of anti-channel catfish IgM mouse monoclonal 9E1 antibody [27] was added at 1:500 dilution in blocking solution. The anti-trout IgM monoclonal antibody was used as an isotype control [11]. After 1 h of incubation at room temperature, plates were washed with PBST and 100 μl of sheep anti-mouse IgG HRP conjugated (GE Healthcare, Pittsburgh, PA) was diluted 1:5000 in blocking solution and incubated for 30 m at room temperature. Plates were rinsed three times with PBST, and 100 μL of 1-Step Ultra TMB-ELISA substrate solution (ThermoFisher, Waltham, MA) was added. The peroxidase reaction was stopped after 20 m with 100 μL of 3 M H_2SO_4 and read spectrophotometrically at 490 nm with a BioTek Synergy H1 plate reader operating under Gen5 software (Winooski, VT).

2.5. 1-D gel electrophoresis and western blot analyses, mass spectrometry

SDS gel electrophoresis was conducted to separate samples of bacterial cellular and ECP fractions using 10% TGX stain-free gels and buffers of the mini-protean system (Biorad, Hercules, CA). We loaded 5 μg of bacterial pellet and 2 μg of ECP onto the SDS gels with the WesternC pre-stained gel marker (Biorad, Hercules, CA) and either stained using Simple Blue Safe (ThermoFisher, Waltham, MA) or transferred onto nitrocellulose membranes utilizing the Transblot system (Biorad, Hercules, CA). For western blot analyses we used a modified float the blot technique to minimize the amount of serum and skin explant medium required. After transfers we first blocked membranes for 1 h at room temperature with PBST with 5% milk. The membranes were washed two times in PBST and prepared for staining. We briefly, cut a piece of parafilm that is slightly larger than the membrane; pipetted some blocking solution onto the staining vessel, and the parafilm was stuck to the container. We made serum (1:200) and skin explant (1:20) dilutions in 1 mL for a full membrane and 0.5 mL for half a membrane. The diluted serum or skin antibody solutions were pipetted onto one end of the parafilm and then using forceps the membrane was laid protein side down onto the diluted solution allowing the solution to wick across the membrane and stained for 1 h at room temperature. The membranes were washed 2 times in PBST separately and then up to two membranes would be stained together with the primary and secondary antibodies. Membranes were washed and incubated with 9E1 mAb (1:500) in blocking solution or with an anti- *E. coli* DNAk monoclonal antibody (8E2/2) (1:2500, Enzo Life Sciences, Farmingdale NY) for 1 h. All membranes were then washed two times and stained with IgG-HRP (1:5000 GE Healthcare, Pittsburgh, PA) and Streptactin HRP (1:5000 Biorad, Hercules, CA) in blocking solution for 30 m. The chemiluminescent signals were developed using ECL plus Western Blotting Substrate according to the protocol (Pierce/ThermoFisher, Waltham, MA) and visualized using a Biorad ChemiDoc XRS+ gel system operating under Image Lab 3.0 software.

For the competitive western blots: catfish serum (1:200) or 8E2/2 (1:2500) antibody solutions were incubated with none, 1 or 10 μg of recombinant *E. coli* DNAk (Enzo Life Sciences, Farmingdale, NY) for 1 h. Blots had already been transferred and blocked. After these initial steps the remainder of the western blot protocol was performed as described above. We then used the Image lab software to conduct densitometry analysis of the different blots from each experiment [41].

Different protein bands in the SDS gels were excised and subjected to LC-MS/MS (liquid chromatography with tandem mass spectrometry) analysis and peptides were identified using the *Flavobacterium columnare* (ATCC49512/CIP103533/TG44/87) reference proteome and outputted into Scaffold 4.4.5 (Proteome Software, Portland, OR, USA).

2.6. Statistics

Survival data was analyzed using Kaplan-Meier log rank survival analysis and differences between groups were determined using unpaired t-tests or Pearson correlation between serum and skin antibody levels. Probabilities of 0.05 or less were considered statistically significant. All statistical tests were performed using GraphPad Prism version 4.0 (San Jose, California).

3. Results

3.1. Post challenge assessment of humoral immune response to F. columnare

We first immunized a group of channel catfish with *F. columnare* isolate LV-359-01 that was cultured under iron-limited conditions, which was previously shown to exhibit slower growth and attenuate virulence [5]. Fourteen days later we challenged control (C) and immunized (IC) fish with wild type *F. columnare* LV-359-01. Kaplan-Meier survival analysis showed that after 4 days post challenge there was no difference in overall survival between the (C, 57%) and (IC, 64%) fish (Fig. 1a). Preliminary experiments in our laboratory indicate that the peak antibody response to both intraperitoneal and immersion immunization to *F. columnare* occurs around 4–5 weeks (data not shown). We subsequently maintained twenty fish from each treatment as well as non-challenged fish (N) for 40 days prior to sampling. To evaluate for the production of *F. columnare* specific IgM antibodies to the bacterial cell fraction, we first screened the serum using an indirect ELISA. The absorbance values (and therefore the relative levels of *F. columnare* antibodies) showed that individual fish generated varying amounts of antibody. The (C) and (IC) fish that had been challenged showed significantly ($P < 0.05$) higher levels of serum antibodies than non-challenged control fish (Fig. 1b); however these values were not significantly different from one another. Fish that were only immunized (I) did mount an antibody response to *F. columnare*. Their mean absorbance values were significantly higher than ($P < 0.05$) that (N) fish and lower than (C) fish, but did not differ significantly from the (IC) pool of fish (data not shown).

We also evaluated the amount of anti- *F. columnare* antibodies from the *in vitro* cultured skin explants, and while there was much less total antibody produced in the skin; ELISA analyses showed that the (C) and (IC) challenged fish had significantly ($P < 0.05$) more IgM antibodies than did non-challenged fish (Fig. 1c), while the (C) and (IC) challenged fish again were not significantly different from each other. Again the mean absorbance values in the (I) immunized only skin were significantly higher than ($P < 0.05$) that (N) skin and lower than (C) skin, but did not differ significantly from the (IC) skin explants (data not shown).

To evaluate whether there was a relationship between the production of antibodies in the serum or skin between the *F. columnare* challenged groups, we next looked for a correlation between these values among individual fish. There was a positive correlation among the production of serum and skin antibodies in the (C) fish ($R^2 = 0.30$, $P = 0.01$), more so in the (IC) fish ($R^2 = 0.87$, $P = 0.001$) (Fig. 1d–e).

3.2. Ab-response patterns among fish after F. columnare infection

To determine the bacterial protein target(s) with which

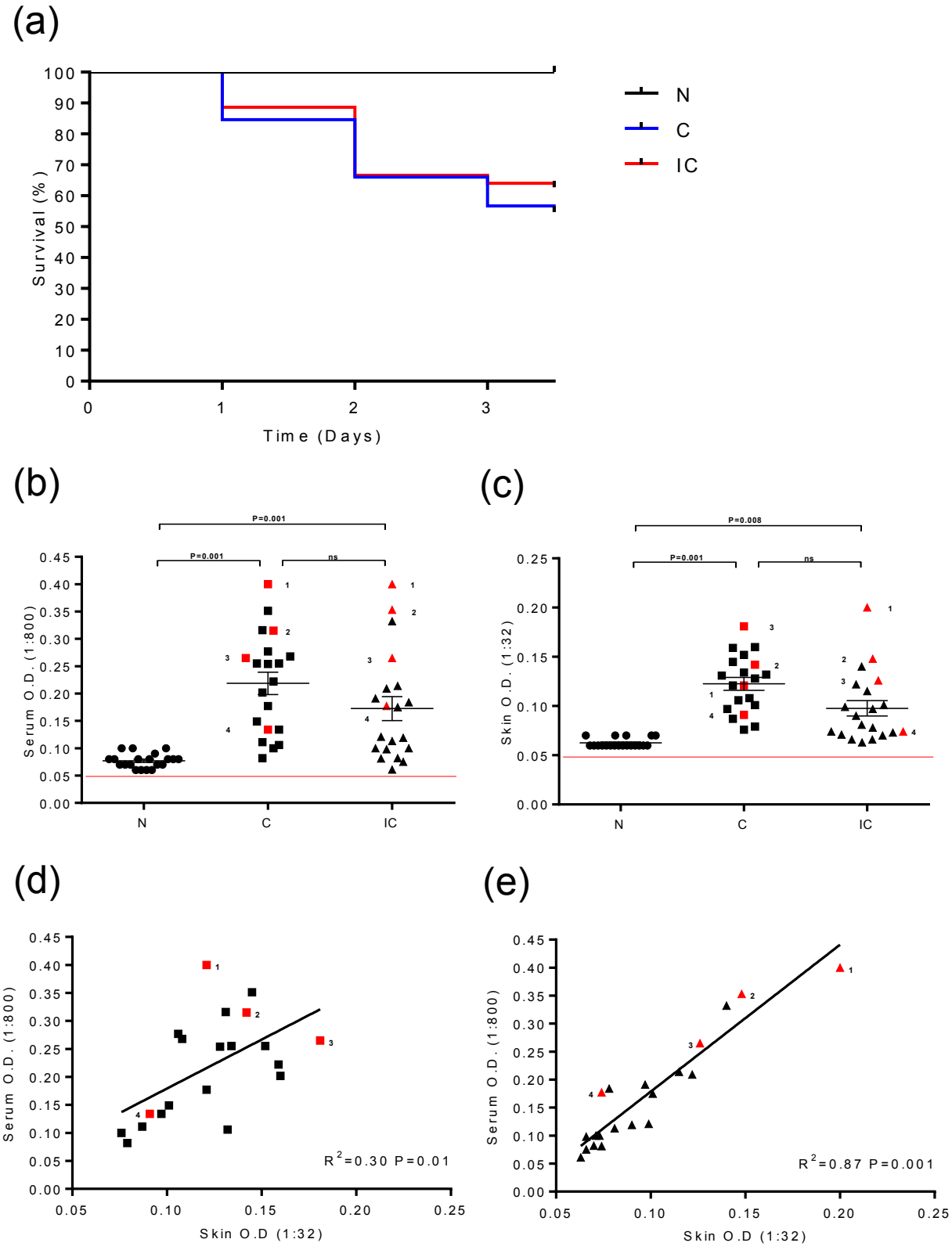


Fig. 1. Adaptive immune response to *Flavobacterium columnare*. (a) Kaplan-Meier survival analysis of non-challenged (N, black) control (C, blue) and immunized (IC, red) groups infected with *F. columnare* LV-359-01. Bacterial cell-induced IgM antibodies in the serum (b) and in the skin (c) determined by ELISA on day 40 post challenge. The mean ± SE for each group is shown as a horizontal line. The red line represents background absorbance observed using an isotype control. (d and e) Pearson correlation between the level of serum and skin IgM antibodies between the control (square) and immunized (triangle) groups. Differences were considered significant ($P < 0.05$). Fish C1-4 (red squares) and IC1-4 (red triangles) represent matched serum and skin samples used throughout the study.

antibodies had been developed, we analyzed the antibody reactivity patterns of individual fish through western blot analyses (C1-4 and IC1-4, Fig. 1b). The fish chosen represent both low and high responders and therefore are an ideal cross section from the two groups. The LV-359-01 and LSU-066-04 bacterial pellets were electrophoresed and blotted with serum from control and immunized fish (Fig. 2a–b). The (C) and (IC) fish exhibited broad antibody specificities to proteins with molecular masses between 30 and 110 kDa. There were no consistent antibody patterns; however some animals (C2 and IC3 or C3 and IC2) showed similar reactivity to the LV and LSU bacterial cell fractions. We also probed the extracellular products (ECP) of the two *F. columnare* isolates and identified a single pattern of binding that was present in all the fish (Fig. 2c–d). An analysis of individually matched skin explants (C1-4 and IC1-4, Fig. 3a–b) revealed very weak binding to the bacterial pellets, however as seen in the serum, all fish showed reactivity to the same single band in the ECP (Fig. 3c–d). Therefore the majority of antibodies developed to *F. columnare* among fish from both groups were reactive to a ~70 kDa band that was present in the LV-359-01 and LSU-066-04 ECP fractions.

3.3. Identification of F. columnare extracellular antigens

To identify proteins associated with the ~70 kDa band in both the LV-359-01 and LSU-066-04 ECP, we gel-excised the bands and used mass spectrometry analysis to resolve different peptides (Fig. 4a). The peptides were compared to a *F. columnare* reference proteome and individual proteins were assigned. The proteins identified between the LV-359-01 and LSU-066-04 isolates were almost identical with minute changes in peptide counts for each protein. The predominant protein was chondroitin AC lyase and accounted for nearly 50% of the total protein in band (Fig. 4b). Additional peptides included those identified with metallopeptidase and gliding motility family proteins. A chaperone protein, DNAk, or heat shock protein was also identified and represented just 5% of the total protein in the 70 kDa band. We concluded that a single or a very few antigenic determinant(s) had likely activated the majority of B-cell responses in these fish.

3.4. Distribution of DNAk epitopes among gram negative bacteria

The identification of heat shock proteins would likely be expected in the cellular fraction, however to identify DNAk in the ECP was rather unexpected. There is a wide array of evidence that shows that heat shock proteins are predominant during an immune response in mammals [56]. This is primarily due to their abundance and the conservation of HSP epitopes across bacterial species. An amino acid alignment showed that *F. columnare* DNAk only shares on average ~60% similarity to *E. coli*, *A. hydrophila* and *E. ictaluri* (Fig. 5a); however there were stretches of conserved amino acids identified in the alignment (data not shown). To establish whether catfish serum antibodies are reactive to DNAk, we conducted western blots using the cellular and extracellular fractions of the different gram negative bacteria. We first assessed for the presence of DNAk in the isolates of *E. coli*, *F. columnare*, *A. hydrophila* and *E. ictaluri* by probing with the monoclonal antibody (8E2/2) which has reactivity to *E. coli* DNAk. 8E2/2 was strongly reactive with a ~70 kDa band in the *E. coli* cellular fraction and less so with the ECP (Fig. 5b). No reactivity was observed in the *F. columnare* cell fraction, but there was moderate cross reactivity in the ECP.

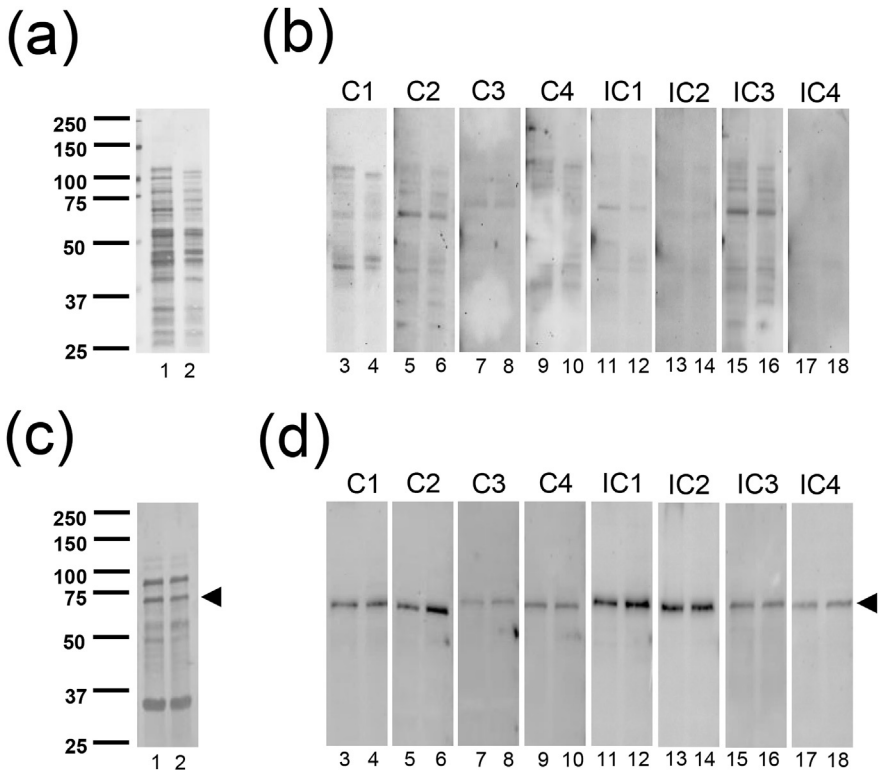


Fig. 2. Serum antibody response to cellular and extracellular fractions of *F. columnare*. (a) SDS gel of *F. columnare* cellular fractions; lane 1, LV-359-01 and lane 2, LSU-066-04. (b) Immunoblot of *F. columnare* cellular fractions; LV-359-01 lanes 3, 5, 7, 9, 11, 13, 15, 17; and LSU-066-04 lanes 4, 6, 8, 10, 12, 14, 16, 18 probed with catfish serum (C1-4; IC1-4). (c) SDS gel of *F. columnare* extracellular fractions; lane 1, LV-359-01 and lane 2, LSU-066-04. (d) Immunoblot of *F. columnare* extracellular fractions; LV-359-01 lanes 3, 5, 7, 9, 11, 13, 15, 17; and LSU-066-04 lanes 4, 6, 8, 10, 12, 14, 16, 18 probed with catfish serum (C1-4; IC1-4). The arrow identifies a 70 kDa band in the extracellular fractions. The pre-stained Western C marker (kDa) was used to estimate molecular mass.

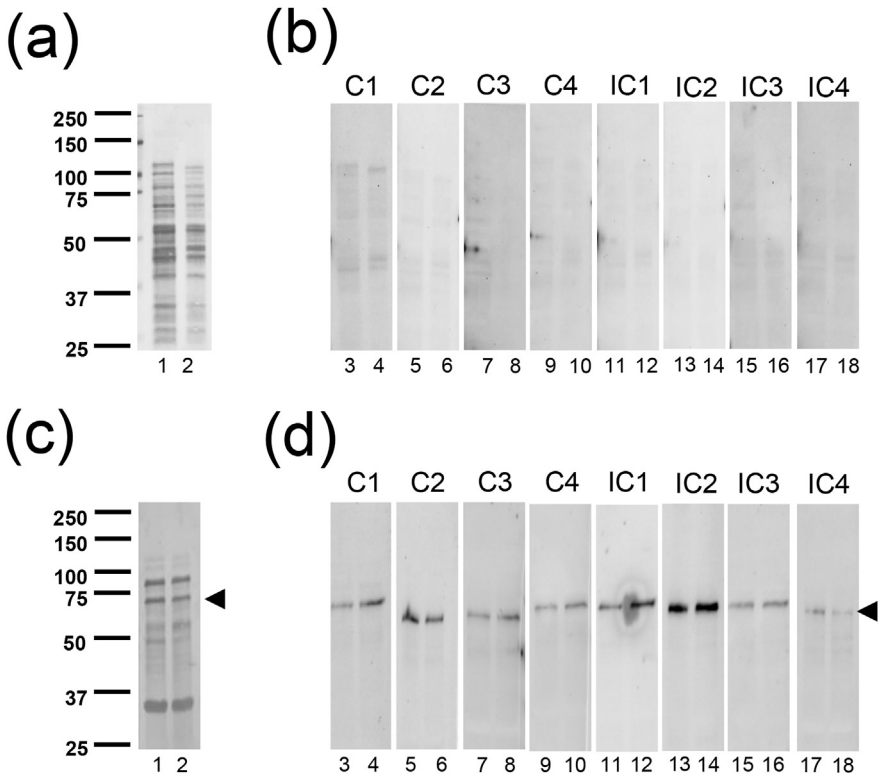
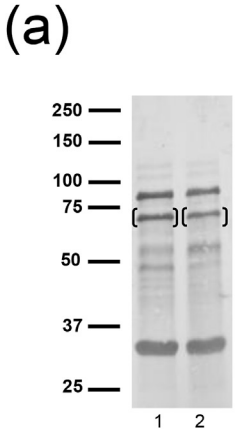


Fig. 3. Skin antibody response to cellular and extracellular fractions of *F. columnare*. (a) SDS gel of *F. columnare* cellular fractions; lane 1, LV-359-01 and lane 2, LSU-066-04. (b) Immunoblot of *F. columnare* cellular fractions; LV-359-01 lanes 3, 5, 7, 9, 11, 13, 15, 17; and LSU-066-04 lanes 4, 6, 8, 10, 12, 14, 16, 18 probed with catfish skin culture medium (C1-4; IC1-4). (c) SDS gel of *F. columnare* extracellular fractions; lane 1, LV-359-01 and lane 2, LSU-066-04. (d) Immunoblot of *F. columnare* cellular fractions; LV-359-01 lanes 3, 5, 7, 9, 11, 13, 15, 17; and LSU-066-04 lanes 4, 6, 8, 10, 12, 14, 16, 18 probed with catfish skin culture medium (C1-4; IC1-4). The arrow identifies a 70 kDa band in the extracellular fractions. The pre-stained Western C marker (kDa) was used to estimate molecular mass.



Protein ID[a]	MW (kDa)	LV-359-01 Spectrum count	LV-359-01 Sequence coverage (%)	LSU-066-04 Spectrum count	LSU-066-04 Sequence coverage (%)
Chondroitin AC lyase	86	234	44	196	45
Polysaccharide lyase family 8	100	81	37	77	38
M13 family metallopeptidase	76	72	49	58	42
Gliding motility lipoprotein	65	26	24	26	22
Chaperone protein (DNAk)	68	22	33	19	26
Uncharacterized protein	36	21	34	33	42

[a] The *Flavobacterium columnare* (strain ATCC 49512 / CIP 103533 / TG 44/87) reference proteome was used to identify peptides.

Fig. 4. Identification of proteins from the extracellular fractions of LV-359-01 and LSU-066-04 isolates. (a) SDS gel of *F. columnare* extracellular fractions; lane 1, LV-359-01 and lane 2, LSU-066-04. The parentheses mark the 70 kDa bands that were gel excised from lanes 1 and 2. (b) Proteins were identified through matching peptides to the *Flavobacterium columnare* (ATCC49512) reference proteome.

A. hydrophila and *E. ictaluri* were weakly cross reactive in the cellular fraction and *A. hydrophila* alone was also weakly cross reactive in the ECP. We then probed the bacterial blots with the catfish serum representing (C) and (IC) fish (C1, C2, IC1, and IC2) and all were cross reactive with a 70 kDa band in the cellular fractions of *A. hydrophila* and *E. ictaluri*, but not with *F. columnare* (Fig. 5c). Among the ECP the serum samples were predominantly strongly reactive to *F. columnare* and much less so to *A. hydrophila* (Fig. 5c). Finally, we probed blots with serum from two pond fish (P1, P2) that had been among the same year class as our other experimental fish, but had been reared in earthen ponds since soon after hatching. These two animals showed no reactivity to *F. columnare* ECP and weak reactivity to *A. hydrophila* cellular fractions and to *A. hydrophila* and *E. ictaluri* ECP fractions (Fig. 5d).

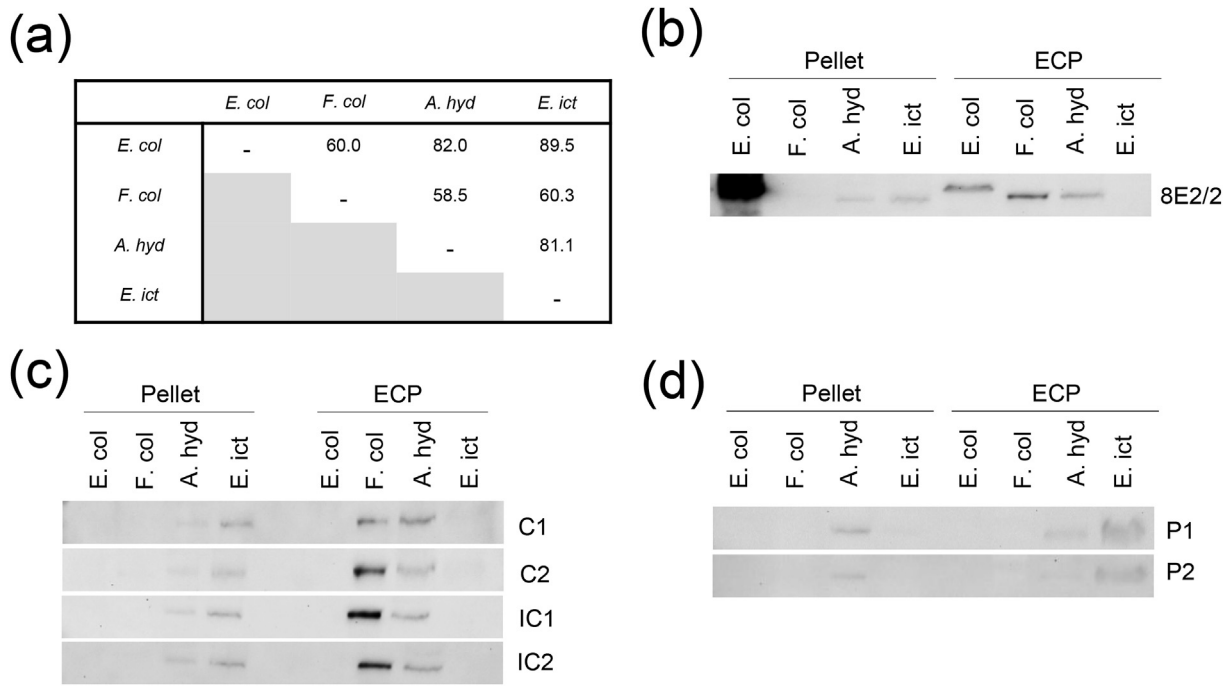


Fig. 5. Immunoreactivity to DNAk protein in gram negative bacteria. (a) Similarity between the amino acid sequences of gram negative bacteria. Abbreviations and Genbank accession numbers for chaperone protein DNAk: E. col (P0A6Y8, K12 strain) *Escherichia coli*, F. col (WP_014165528) *Flavobacterium columnare*, A. hyd (KLV44233) *Aeromonas hydrophila*, E. ict (C5B7L7) *Edwardsiella ictaluri*. (b) Immunoblot of gram negative bacteria cellular (pellet) and extracellular (ECP) fractions using the anti-DNAk monoclonal antibody (8E2/2) that correspond to a 70 kDa band. (c) Immunoblot of gram negative bacteria cellular (pellet) and extracellular (ECP) fractions using catfish serum (C1-2; IC1-2) that correspond to 70 kDa band. (d) Immunoblot of gram negative bacteria cellular (pellet) and extracellular (ECP) fractions using pond catfish serum (P1-2) that correspond to a 70 kDa band.

3.5. DNAk epitopes dominate the antibody response to F. columnare

Following the discovery of anti-DNAk antibodies in (C) and (IC) catfish serum, we next sought to determine whether these anti-DNAk antibodies represented a major fraction of the total antibodies produced. To this end we utilized competitive western blot protocol employing recombinant DNAk (rDNAk) protein and incubated it with 8E2/2 antibody prior to staining an *E. coli* ECP blot. We then developed and assessed the relative intensity of the chemiluminescent signal to determine if the rDNAk had blocked binding to the extracellular DNAk [41]. In a blot without rDNAK we detected a baseline signal of 5.4×10^6. After the addition of 1 μg rDNAK a ~6 fold (0.83×10^6) loss in signal was observed, followed by a ~60 fold (0.085×10^6) loss in signal when 10 μg of rDNAK was incubated with 8E2/2 (Fig. 6a–b).

Next we incubated rDNAK with catfish serum antibodies and again looked to assess the relative intensity of the different blots. The signals among the different serum samples varied, however a mean baseline signal of 3.9×10^5 was followed by a ~2 fold (1.8×10^5) loss in signal when 1 μg rDNAK was added and a ~3 fold (1.3×10^5) loss in signal with 10 μg of rDNAK (Fig. 6a,c).

4. Discussion

The current study sought to evaluate differences between the humoral immune responses in channel catfish that had been immunized as compared to unimmunized fish after an active challenge with *F. columnare*. It became apparent early on that our immunization protocol had not increased protection against columnaris disease, as our survival curve showed no difference between (C) and (IC) fish. We therefore instead sought to compare the individual B-cell responses to *F. columnare* between the two groups. The evaluation of IgM antibody production and the characterization of bacterial protein targets revealed a primary antibody response to bacterial extracellular proteins in all fish. The heat shock protein DNAk was identified as the target for cross reactive antibodies that bind to this protein in different gram negative bacterial species. These results allow for some conclusions to be made about the catfish B-cell response to *F. columnare*.

Our initial assessment of IgM antibody production showed that immunization with *F. columnare* LV-359-01 that was cultured under iron-limited conditions did elicit a humoral immune response. There was however no difference in survival or overall IgM antibody production between the (C) and (IC) treatments after bacterial challenge. We did observe positive correlations between antibody production in the serum and skin, and that each group had low and high immune responders to *F. columnare* [37]. A higher R^2 value in the (IC) fish suggests that the (C) fish had only just begun to undergo systemic and mucosal B-cell responses, whereas the immunized fish had likely undergone a more disseminated immune response implying that some of the B cells had previously responded to *F. columnare*. The time interval of immunization and the dose of bacterins definitely play a role in the intensity of the overall adaptive immune response [29,43]. Clearly this is an area that will need to be further studied if a protective immune response to *F. columnare* is to be achieved.

Previous reports have examined the antibody response to *Flavobacterium* sp. and *F. columnare* and have used multiple approaches to identify antigens [17,21,24,38,51,53]. These approaches have generally relied on pooled antiserum and whole bacterial lysates to identify these targets. To better compartmentalize and allow for a more detailed analysis of the IgM antibody response, we prepared separate cellular and extracellular fractions of *F. columnare* isolates. After screening multiple control and immunized individuals, we identified that there were serum and skin antibody responses to the bacterial cellular fractions as opposed to

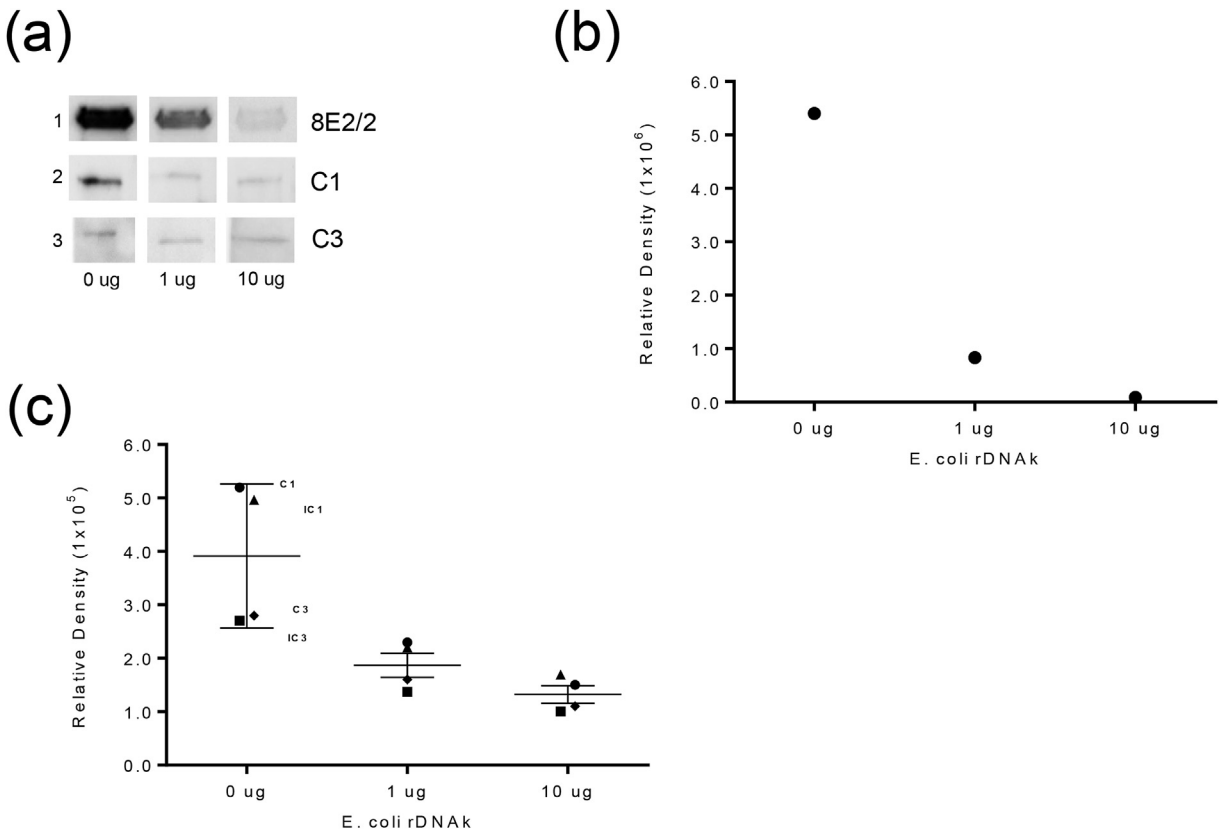


Fig. 6. Competitive inhibition to extracellular DNAk protein. (a) Immunoblot of extracellular (ECP) fractions after the addition of recombinant DNAk; lane 1, *E. coli* using the anti-DNAk monoclonal antibody (8E2/2) and lanes 2–3, *F. columnare* using catfish serum (C1 and C3) that correspond to the 70 kDa band. (b) The relative density of signals corresponding to a 70 kDa band by 8E2/2 spiked with or without recombinant DNAK prior to staining of the blots. (c) The relative density of signals corresponding to a 70 kDa band by different catfish serum blots spiked with or without recombinant DNAk prior to the staining of blots.

the extracellular which seemed to be essentially monovalent. Unlike the cellular fractions that have hundreds of different expressed proteins [12,23]; the extracellular fractions have much fewer immunogens, but have been sought for use in the development of vaccines [52]. Our analysis found that a single or a very few antigenic determinant(s) were likely immunodominant and initiated the bulk of B-cell responses in these individuals [45,55].

Heat shock proteins are widely disseminated and represent a highly conserved family of proteins [57] and there is an array of evidence suggesting that heat shock protein are prominent in the adaptive and cellular immune response in mammals [56]. The immunogenic potential of heat shock proteins to activate a B-cell response in fish was previously demonstrated in grass carp where IgM and IgZ antibodies, respectively were generated against cellular DNAk and GroEL chaperonins [24]. We had made a novel observation in that we identified DNAk in the extracellular fraction of both *F. columnare* isolates through our mass spectrometry analysis and was further shown to solely be present in the ECP when we probed with the anti-DNAk monoclonal antibody (8E2/2) and catfish antiserum. There is a precedent for identifying DNAk in the ECP as observed by a group studying the proteome of *Aeromonas salmonicida*. Their identification was among a long list of cellular proteins that are “moonlighting” in the ECP, and they suggested perhaps that DNAk would be a viable vaccine candidate [44]. We also show that 8E2/2 and more importantly catfish antiserum generated against *F. columnare* are reactive to the DNAk proteins of several gram negative bacterial fish pathogens. While 8E2/2 only recognizes a single conserved epitope, which has to be present in all four bacteria; it seems more likely that the polyclonal catfish antibodies are reactive to different epitopes present in all the gram negative bacteria opposed to a conclusion that these individuals made antibodies against all four bacterial species separately. The anti-DNAk responses in two irrelevant pond fish that have weak reactivity to *A. hydrophila* and *E. ictaluri* but not *F. columnare* further suggests that they separately recognized conserved epitopes not found in the *F. columnare*. This too has been shown in other gram negative bacteria (*Salmonella, Citrobacter, Shigella* and *Vibrio*) where different monoclonal and polyclonal antibodies show variable reactivity to their DNAk protein counterparts [13].

We show that the addition of recombinant DNAk protein blocks binding to the *F. columnare* extracellular DNAk through its competition for the antigen binding sites of the 8E2/2 anti-DNAk monoclonal antibody. This made since considering the rDNAk protein is the immunogen used to generate the 8E2/2 anti-DNAk monoclonal antibody. The immunogen potential of *F. columnare* DNAk was also validated because at least half of the antibodies tested in several individuals demonstrate that they were likely produced to this single antigen. This potential had not been lost on others who looked to the HSPs 60 and 70 proteins in *Flavobacterium psychrophilum* as vaccine targets [32]. They concluded that despite a high immunogenicity there was no protective capability to the antibodies that were made. We can't completely rule out that these anti-DNAk antibodies afford some protection to these animals; however based on the literature it seems unlikely.

Chaperone proteins function through stabilizing other proteins to ensure correct folding or refolding proteins that were damaged by cell stress [57].

As briefly mentioned above some bacterial proteins can take on

other functions than those generally ascribed to them and are referred to as moonlighting proteins. A more traditional role for the DNAk protein in aiding the virulence of different bacterial pathogens has been described [6,7,40]; however moonlighting likely would take them out of these normal roles [46]. One early study demonstrated that *Mycobacterium tuberculosis* could have enhanced virulence through the binding the human plasminogen protein to its outer membrane bound DNAk and activate it into enzymatically active plasmin [49]. Alternatively others have speculated that the identification of cytosolic proteins in the ECP of both gram positive and negative bacteria is likely due to cell lysis and not necessarily to a mechanism of the normal life cycle of bacteria [16]. So what is the role of this highly immunogenic extracellular *F. columnare* DNAk protein? Other studies will need to characterize responding B-cell populations and identify specific epitopes and elucidate whether there is a functional role for *F. columnare* anti-DNAk antibodies in immunity against columnaris disease [19,33].

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